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(54) Title: USE OF POLYMORPHISMS AS A PREDICTOR OF DRUG-RESISTANCE MUTATIONS

(57) Abstract

Significant drug-resistance mutations in HIV are preceeded by the development of polymorphisms in the bases located on either side of the site of the drug-resistance mutation, and similar polymorphisms are likely to occur prior to other drug-resistance mutations. A method is provided for detection of polymorphic mutations which provides an indication of an increased risk of an imminent drug-resistance mutation, which can be considered in determining whether or not to maintain a patient on a given drug-therapy.

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USE OF POLYMORPHISMS AS A PREDICTOR OF DRUG-RESISTANCE MUTATIONS

This application relates to a method for predicting drug-resistance mutations by detection of polymorphisms.

In the treatment of infectious diseases and cancer with anti-microbial (including anti-viral) drugs or chemotherapy agents, a significant problem which must be contended with is the development of drug resistance after a period of time. Such drug resistance arises as a result of mutations within the genome of the target virus, organism or cell, where the mutant form is able to compete effectively with the wild-type virus, organism or cell in the presence of the drug or chemotherapy agent. This leads to selection of the mutant form, such that it eventually may become the dominant species present.

The development of drug-resistance mutations have been observed for other viruses, bacteria and in cancer cells. In each case, these mutations at a minimum reduce the number of therapeutic options, and may be so significant that they effectively eliminate any possibility of effective treatment. For example, the hazards of induced drug resistance are clearly evident from consideration of various drug resistance mutations which are observed after nucleoside therapy for treatment of human immunodeficiency virus (HIV) infections.

The Human Immunodeficiency Virus (HIV) has an extraordinary capacity for replication and mutation. An estimated 10^9 -10^{10} viral particles are produced each day within the HIV infected host. Each of these viruses theoretically can infect and destroy a CD4 bearing lymphocyte. The rapid replication of the virus offers the opportunity for mutation and the inexact nucleotide reading and transcription of the viral RNA dependent DNA polymerase (reverse transcriptase) further contributes to the problem. It is estimated that HIV reverse transcriptase makes an error once for each one thousand base pairs of RNA transcribed. Thus the HIV genome, with 10,000 base pairs of RNA, offers the opportunity for ten mutations to occur with each viral particle produced. Thus, in theory, each infected person may harbor countless potentially resistant mutants despite never having been treated with antiretrovirals.

Persistent HIV infection can be sustained for many years while the immune system is capable of replacing CD4 T cells as rapidly as the infection destroys them. Studies have shown that higher viral load (which by inference means greater virus production) is associated with poorer prognosis. This is believed to be because the higher viral production results in more rapid exhaustion of the capacity of the immune system to replenish the CD4 T cells. As CD4 cells are depleted, the symptoms of HIV appear such as mucosal candidiasis, oral hairy leukoplakia, chronic unexplained fever, night sweats, and susceptibility to various opportunistic infections such as pneumonia, shingles, tuberculosis, cryptococcosis, cytomegalovirus, and MIA/MAC.

Viral load is currently believed to be the best available surrogate marker of HIV disease progression. One reason for an increase in viral load is the emergence of drug resistant mutations which render the subject's therapy ineffective and may therefore require a change of medication to maintain lower levels of viral load and improved prognosis.

Multiple studies have now inextricably linked prognosis with viral burden in HIV infected persons. The goal of HIV therapy is the suppression of viral replicative ability to zero, that is, to make it impossible for the virus to reproduce viable, infective progeny. The tools used to achieve this goal are the 12-15 antiretroviral drugs currently available by prescription or compassionate use pathways. However, the introduction of new drugs or drug treatment strategies over the past decade has been shortly followed by the emergence of resistant virus. HIV variants with decreased susceptibility to zidovudine (AZT) were first reported in 1989. Since this first report the use of virtually every available antiretroviral agent has been accompanied by the emergence of HIV variants resistant to drug concentrations initially needed to inhibit replication and growth. Recent reports of the transmission of resistant virus and the emergence of resistance in more than 50% of highly active antiretroviral therapy treated persons underscore the need to define a more efficient, less time consuming technology for HIV characterization.

Resistance may emerge because current therapies may not achieve total suppression of viral replicative ability. As previously stated, a complex "library" of

mutant virus is likely present in each HIV infected person. Untreated, these mutants are suppressed by the superior replicative ability of the "wild type" virus. However, no matter how effectively a drug suppresses replications of wild-type virus, this effectiveness is limited by the frequency of resistant mutants present at the start of treatment and the ability of these to replicate in the presence of drug. Drugs may suppress replication of some genetic variants in the complex HIV-1 population; however, more "fit" viruses may continue to replicate. Combination therapy including protease inhibitors may suppress HIV below the current level of detection for extended periods of time. However, if not completely suppressed, these more "fit" viruses continue to replicate and eventually emerge as the predominant variant.

In vitro studies have been predictive of the nature and pattern of clinical resistance. The analysis of HIV arising in individuals treated with specific drugs or drug combinations has led to the recognition of specific genotypes which confer clinical resistance. One benefit of this information is the ability to rapidly detect the emergence of drug resistant genotypes using highly sensitive molecular tools. However a paucity of information exists on the clinical utility of genotypic resistance testing in planning and managing clinical antiretroviral therapy.

One such HIV genotype testing system has been developed by Visible Genetics, Inc. (Toronto, Canada). Pre-clinical test data indicate that the product (HIV Genekit) should be able to reliably detect drug-resistant mutations in clinical samples, including samples with heterozygotes present. These data suggest that HIV genotype analysis may have a role to play in the assessment of a subject's response to antiretroviral therapy by identifying the presence of any drug-resistant mutations and may be able to provide a treating physician with informed and objective antiretroviral therapeutic choices.

Because of the potential significance of induced mutations that confer drug resistance, it would be highly desirable to be able to predict when such a mutation was about to occur. A patient might then be taken off of the mutation-inducing therapy and switched to a different therapy as medically appropriate. To date, however, no effective method for predicting when a drug-resistance mutation

will occur has been identified. It is an object of the present invention to provide such a method.

SUMMARY OF THE INVENTION

The present invention is based upon the surprising discovery that significant drug-resistance mutations in HIV are preceded by the development of polymorphisms in the bases located on either side of the site of the drug-resistance mutation, and the resulting understanding that similar polymorphisms are likely to occur prior to other drug-resistance mutations. Detection of these mutations therefore provides an indication of an increased risk of an imminent drug-resistance mutation, which can be considered in determining whether or not to maintain a patient on a given drug-therapy.

Thus, the present invention provides a method for detecting a polymorphic mutation effective to act as a predictor of subsequent induction of a drug-resistance mutation at a defined site in a genetic sequence of interest obtained from a source of sample, comprising the steps of:

(a) obtaining a test sample containing the genetic sequence of interest from the source; and

(b) comparing the bases in a region of the genetic sequence of interest in the test sample located on at least one side of the defined site with the same bases in a standard sequence or a sequence determined from a prior sample obtained from the same source, wherein a difference in the bases between the test sample and the standard sequence or prior sample is indicative of an increased risk of subsequent induction of a drug-resistance mutation at the defined site. The most significant bases are generally those that are located near the defined site, i.e., within about 138 bases, or more preferably within about 12 bases of the defined site. The comparison of the bases in the sample and standard genetic sequences is preferably performed by sequencing of the gene through at least 3 base region immediately adjacent to the site of the rug-resistance mutation. However, other techniques including the use of hybridization probes may be employed to detect polymorphisms..

DETAILED DESCRIPTION OF THE INVENTION

Definitions

While the terms used in this application are intended to have their ordinary meaning as understood by persons skilled in the art, the following definitions are provided to avoid any ambiguity.

Derived from: The genetic materials which are tested in the method of the present invention are "derived from" a particular source. As used in the specification and claims hereof, the term "derived from" encompasses any means of obtaining a sample which is indicative of the genetic sequence of the indicated source, including without limitation amplification techniques such as PCR or LCR amplification and cloning and expression of a desired sequence. In the case of a virus such as HIV, the genetic material evaluated may be viral nucleic acids obtained directly from isolated virions or it may be host nucleic acid copies of viral genes that have been incorporated into the host's genetic material.

Drug-resistance mutation: As used in the specification and claims of this application, the term "drug-resistance" mutation refers to a substitution mutation (e.g., a point mutation) in which one or more bases are replaced with other bases to produce a phenotype which is resistant to a therapeutic drug.

Polymorphism or ***Polymorphic mutation:*** As used in the specification and claims of this application, the term "polymorphism" or "polymorphic mutation" refers to a variation in the sequence of a gene from the wild-type sequence that does not confer any significant difference in the phenotype of the individual. In many cases, a polymorphic variation in the genetic code will not change the amino acid associated with the codon, and thus no phenotypic variation will occur.

Genotyping studies of 57 patients undergoing treatment for HIV infection has revealed the formation of polymorphic mutations in the regions adjacent to the site of an induced drug-resistance mutation at a time prior to the appearance of the drug-resistance mutation in genotyping results, and that in many cases, the mutations observed were the same across many of the patients.

For example, a important drug-resistance mutation which occurs in HIV is a mutation at codon 36 of the HIV protease that can be caused by treatment with ritonavir and nelfinavir which changes the codon from ATG to ATA. This results in a change from methionine to isoleucine in the expressed protease. In 57 of 57 patients where this mutation was eventually observed, it was found that earlier genotyped samples had included a polymorphic mutation in codon 35 or 37. Two particularly common polymorphisms are mutations at codon 37 from AGT to AAT or GAT (41 patients) and at codon 35 from GAA to GAC or GAT (21 patients). Note that some patients developed both of the polymorphisms prior to the appearance of the drug resistance mutation at codon 36.

Similarly, within the HIV reverse transcriptase gene, the drug AZT (zidovudine) is known to induce a drug-resistance mutation at codon 215, in which ACC (coding for threonine) is changed to TTC (coding for phenylalanine) or TAC (coding for tyrosine). In 100 of 100 patients where this mutation was eventually observed, it was found that earlier genotyped samples had included a polymorphic mutation in codon 214, in which the normal sequence CTT (coding for leucine) was changed to TTT or TTC (both coding for phenylalanine).

Other predictive mutations for HIV are listed in Table 2.

While not intending to be bound by any particular theory of operation, it is believed that these results can be explained on the basis of a change in the conformation of the secondary structure of RNA that accompanies the polymorphic mutation and that increases the likelihood of a mutation occurring at the drug-resistance site. It has been previously observed that antiviral drug resistance mutations in HIV Type 1 reverse transcriptase occur in specific structural regions of the viral RNA which are generally non-helical in structure (i.e., loops, bulges and bends). Shinazi et al., *Antimicrobial Agents and Chemotherapy* 38: 268-274 (1994). Similar observations have been made for the HIV *env* protein. Le et al., *Nucl. Acid Res.*, 17: 3275-3288 (1989). It appears that the predictive polymorphisms located near the drug resistance site alter the interaction of the non-helical region to impair (or to further impair) the ability of the region to interact with the polymerase enzyme, thus increasing the likelihood of an error occurring during transcription. As used in

the specification and claims hereof, a polymorphism is "near" a mutation site if an alteration at the site of the polymorphism is able to cause an alteration of the secondary structure at the site of the drug resistance mutation. It will be apparent that the precise number of bases which may fall within this range will depend on the secondary structure of any given RNA molecule. In general, however, the number of bases of significance will be within about 138 bases of the drug resistance mutation site, and preferably within about 12 bases.

This model for the observed behavior in HIV supports the conclusion that polymorphic mutations will occur as valid predictors of other types of drug-resistance mutations which occur in similar conformational locations in RNA. Thus, the method of the invention should be useful for prediction of drug-resistance mutations in other virus besides in HIV such as HBV, HCV, CMV and HTLV1, in bacteria such as *Mycobacterium tuberculosis*, and in cancer cells which have known drug resistance mutations. See, Tatti et al., *Int'l Antiviral News* 6:6-9 (1998), cataloging mutations in CMV; Bartholomeusz et al., *Int'l Antiviral News* 5: 123-124 (1997) identifying drug resistance mutations in HBV; Musser et al. *J. Infect. Dis.* 173: 196-202 (1996) and Williams et al., *Antimicrob. Agents Chemother.* 38: 2380-2386 (1994) discussing drug resistance mutations in *Mycobacterium tuberculosis*; and Aas et al., *Nature Medicine* 2: 8211-814 (1996) describing relationship between mutations in the p53 tumor suppressor gene and resistance of breast cancer to chemotherapy.

In accordance with the basic method of the invention, a test sample is obtained which contains the genetic sequence to be evaluated for the presence of predictive polymorphic mutations. Suitable samples will include any material which contains a sufficient amount of genetic material for analysis, and include without limitation samples of blood, urine, sputum, saliva, vaginal or cervical scrapings and biopsy tissue specimens.

The genetic material in the sample is evaluated to compare the sequence of bases near a known site for a drug-resistance mutation with a standard sequence of bases. The bases analyzed may be those which are immediately adjacent to the known site for the drug-resistance mutation, or (in the case where the predictive

mutation is separated from the site by intervening codons) the bases may be somewhat removed from the site of the drug-resistance mutation.

The comparison of the bases near a known site for a drug-resistance mutation in the sample with a standard sequence of bases can be accomplished by sequencing the relevant gene, or that portion of the gene which include the mutation site. For example, in the case of the initial genotyping procedures which led to the discovery of the present invention, sequences of the complete HIV pol gene (including the reverse transcriptase and protease regions) were obtained using a TRUGENE™ HIV Genotyping GENEKIT™ (Visible Genetics Inc., Toronto, CA) and the OPENGENE™ sequencing platform (Visible Genetics Inc., Toronto, CA). The TRUGENE HIV genotyping kit is described in US Patent Application No. 08/938,641, which is incorporated herein by reference. Plasma extraction was carried out using ultracentrifugation and a modified column purification method as shown in the attached protocols. The sequences were then determined and aligned using a nucleotide alignment program (GENEWORKS, Intelligenetics, Inc.). This alignment highlighted polymorphisms present in the samples, and could be used to show the consistent development of polymorphic mutations prior to the development of drug-resistance mutations.

The results of the preliminary retrospective study for 2 of the 57 HIV patients evaluated are summarized in Table 1.

TABLE 1

Patient ID.	Codon of Drug Resistance Mutation (genotyping date when first observed)	Codon of Precursor Polymorphic Mutation (genoptying date when first observed)
KK1	RT215; ACC-> TCC (1/98) RT215; ACC-> TAC (3/98)	RT214; CCT-> TTT (11/97) RT214; CCT-> TTC (1/98)
KK1	PRO36; ATG->ATR (3/98)	PRO37 ; AGT-> AAT (1/98)
TC1	PRO36; ATG->ATA (11/97)	PRO35; GAA->GAT (9/97)

It is noted that some polymorphic changes are not random, and that many patient genotypes had identical variation at specific codons located near resistance-associated mutations. Furthermore, most resistance mutations were accompanied by specific polymorphic changes. When early treatment samples were subjected to clonal analysis (n=20), no mutations were identified other than polymorphic variations. This observation of sequential early patient samplings demonstrated that the previously observed polymorphic changes consistently proceeded true resistance mutations.

Other examples of predictive polymorphisms which anticipate the appearance of drug resistant forms of HIV are summarized in Table 2.

Table 2

Resistance Mutation	Drugs	Predictive Polymorphisms
protease L90M (TTG->ATG)	Ritonavir, Nelfinavir, Indinavir, Saquinavir	91 ACT->ACG or ACC 93 ATT->CTT 94 GGT->GGC
protease A71V (GCT->GTT)	Ritonavir, Nelfinavir, Palinavir, A-77003, BILA 1906 BS, Indinavir	68 GGA->GGG or GAT 70 AAA-> AAG 72 ATA->GTA or ATC->CTA
rt or protease M41L (ATG->TTG or CTG)	Zidovudine (AZT)	87 TTC->TTT 43 GAA->GAC or GAT 45 GGG->GGA 44 GAA-> GAC or GAT 42 GAA->GAG

The determination of predictive polymorphic mutations for other known drug-resistance mutations can be determined using this same methodology without experimentation. Given the information provided in this disclosure, the process of reviewing existing or newly generated data to look at the time sequence of

mutational events in the regions adjacent to other drug-resistance mutations, and thus to identify polymorphic variations associated with subsequently occurring drug-resistance mutations, is essentially a clerical process which may be facilitated by the use of computer software.

While sequencing the region adjacent to a defined site associated with a drug-resistance mutation is the most general method for the detection of predictive polymorphic mutations, it will be appreciated that other methods can also be used for purposes of comparing the sequence of genetic material in a sample with a standard. For example, hybridization probes may be used to bind to the region of interest on either side of the site of the resistant mutation. If the sequences of expected polymorphisms have not been determined for a given resistance mutation in a given gene, or if that mutation does not have the same level of consistency in the nature of the polymorphisms as observed for HIV, then the hybridization probe used would suitably be perfectly complementary to the wild-type sequence adjacent to the resistance-mutation site. Failure of such a probe to hybridize with sample nucleic acids under stringent conditions (detected using any of the many and conventional techniques for detecting hybridization probes which are known in the art) would be indicative of the presence of a polymorphism in the region spanned by the probe and thus of an increased risk of the near-term formation of a drug-resistance mutation.

If the sequences of the expected polymorphisms are known and consistent as in the case of the polymorphisms found to be predictors of the formation of drug-resistance mutations at codon 214 of the HIV reverse transcriptase gene or codon 36 of the HIV protease gene, then a hybridization probe which is complementary to the sequence including the polymorphism could be employed. In this case, binding of the probe to sample nucleic acids would be indicative of the presence of a polymorphism in the region spanned by the probe and thus of an increased risk of the near-term formation of a drug-resistance mutation.

In some cases, it may also be the case that the introduction of the polymorphism will create or destroy a restriction site. In this case, where expected polymorphisms are known and characterized, restriction fragment length polymorphism and similar techniques can be used to assess the presence of the

polymorphism and hence whether there is an increased risk of the near-term formation of a drug-resistance mutation.

It will be appreciated that other techniques can also be used to compare the sequence of the sample nucleic acids to a standard sequence, and that this application is not intended to be limited to the use of any particular technique, or to the use of those techniques specifically named herein. It will further be appreciated that the different techniques can be used in combination, for example in a hierarchical assay of the type described generally in US Patent No. 5,545,527 and International Patent Publication No. WO 96/00761 which are incorporated herein by reference. In addition, measurements of viral load plus sequencing can be performed on a routine basis to monitor a patient for predictive polymorphisms and viral load. This will allow better management of the patient's HIV infection. A suitable procedure for determining viral load and sequencing is disclosed in US Patent No. 5,795,722, which is incorporated herein by reference.

If a polymorphism is detected, this result is reported to the physician treating the patient so that an appropriate course of continued treatment can be determined. In some cases, it may be that the appearance of predictive polymorphic mutations will argue in favor of a cessation of therapy, or of a shift to treatment with a different therapeutic agent. It is outside the scope of the present invention to take the place of the physician making decisions with respect to individual patients, where issues such as drugs previously used and the availability of and tolerance to alternative treatments may play a role.

The method for the invention may suitably be practiced using a kit. While any kit which contains suitable sequencing primers may be used for sequencing through the regions having predictive polymorphisms for a resistance-mutation, it will be appreciated that the present invention gives significance to regions of the genetic sequence which had not previously been understood to be therapeutically or diagnostically relevant. Thus, kits which are constructed to permit specific evaluation of these regions, for example using hybridization or other probes which specifically bind to the region adjacent to the resistance mutation site specific in its wild type of

polymorphic form and which are otherwise adapted for the performance of the method of the invention are considered to be within the scope for which protection is sought.

EXAMPLE 1

The following protocols and procedures can be followed for determination of the sequence of HIV mutations in the RT or Protease gene:

Sample Log-in Standard Operating Procedure (S.O.P.) IA

General considerations for sample receipt and initial preparation:

- Decontaminate sample with 70% ETOH and look for signs of leakage. Make notes pertaining to sample, i.e. Plasma (thawed or frozen) or whole blood.
- Store whole blood samples in 4°C cooler. Samples that arrive as plasma should be stored in the appropriate box at -70°C.
- Log samples in the receipt log. This is done in writing on log form located on the -20°C freezer. Always include initials.
- Give samples an APSCI code as follows:

  1) Physician Initials

  2) Patient Initials + number (patients with identical initials will have consecutive numbering) i.e. JB1 or JB2.

  3) Sample number designation (this is a consecutive number of a received sample from an individual patient) i.e. 0001, 0002, 0003, 0004..., etc.
- Sample sheet (Requisition form from physician) should be decontaminated and placed in Logged Requisition Form Book. The log book is alphabetized by Physician's last name.

Whole Blood Preparation S.O.P. IIA

- Patient whole blood samples are spun at maximum speed in a blood centrifuge (Fisher Brand Centrific model 228) for no less than 15 minutes.
- Collect plasma excluding red and white blood cells and transfer to appropriate tube. For storage transfer plasma to Sarstedt screw top 2.0 ml tube (catalog # 72.693.005) and place at -70° C until use. For direct RNA isolation follow

S.O.P. IIB. Be sure to follow APSCI coding procedure as described in "Sample Log-in" S.O.P. IA).

PELLETING VIRUS & LYSIS S.O.P. IIB

- Directly transfer plasma to Beckman high speed 1.5 ml tubes (Microfuge®Tube Polyallomer catalog # 357448 with Beckman rotor F3602).
- Mark outside position of each sample tube with an orientation mark (This will assist in identifying the viral pellet).
- Spin tubes containing plasma at 45,000 x g maximum for 60 minutes (Beckman Avanti™ centrifuge).
- Using blood bank polyethylene transfer pipet (Fisher catalog # 13-711-49) and non-barrier p200 (Rnase Free) tip remove plasma. Be sure not to disrupt viral pellet.
- Add 200μl of sterile Rnase free phosphate buffered saline (PBS) to the viral pellet. Pipette gently to resuspend viral pellet. (If more than 1 tube was used for an individual patient the PBS/viral resuspension can be added to additional tube to batch concentrate virus).
- Go to Standard BMB (Boehinger Mannheim Biochemical) High Pure Viral RNA Kit (cat # 1858882)***SEE PROTOCOL: BMB VIRAL RNA KIT

Supplies & Reagents not supplied in BMB kit

- PBS solution (Gibco BRL 0.01M pH 7.40)
- Beckman tubes
- polyethylene transfer pipet
- non barrier pipette tips

BMB HIGH PURE VIRAL RNA KIT

Working solution:

Option 1: Resuspend polyA RNA carrier (vial 2) with 2 ml of Binding Buffer (1 ml from each bottle), then aliquot 1 ml to each Binding Buffer bottle.

Option 2: Take 40 μl aliquot of polyA RNA carrier (vial 2) out of -20°C and mix with 5 ml of Binding Buffer (for 10 samples; good for 3 months at room temperature).

1. Label all tubes.

2. To 200μl serum or plasma sample add 400μl Working solution, mix by pipetting SLOWLY or TOUCH VORTEX.

3. Add above sample to *High Pure* filter tube containing collection tube, and centrifuge for 15 seconds at 8,000 x g.

4. Discard collection tube with flowthrough.

5. Transfer *High Pure* filter containing sample to new collection tube and add 450 μl wash buffer then centrifuge for 15 seconds at 8,000 x g.

6. Repeat steps 4 and 5.

7. Transfer *High Pure* filter containing sample to new collection tube and centrifuge for 10 seconds at 13,000 x g to dry.

8. Transfer *High Pure* filter containing sample to sterile 1.5 ml microcentrifuge tube.

9. Add 50 μl of elution buffer and let it sit for 1 minute.

10. Elude RNA by centrifuging for 1 minute at 8,000 x g.

C-DNA & RT-PCR Amplification S.O.P. IIIB

- Fill out appropriate RT-PCR worksheet with sample orientation and numbering. (Note: you must include appropriate controls for reagents and controls with each experiment.)

- Clean RNA hood using 10% bleach. Be sure to clean afterwards with 70% ETOH. (Note: Pipettes in RNA Hood are for RT-PCR and v-RNA handling only.) Pipettes must be cleaned internally with Rnase Away cleaner 2X weekly. Pipettes should be cleaned externally with 10% bleach after every use, and cleaned with Rnase Away 2X weekly.

- Follow Method cDNA & PCR for HIV-1 Genotyping S.O.P. IV

Thermocycler perimeters vary from Region to Region. Be sure you are in the right program.

S.O.P. IV cDNA & PCR Protocol

Ready To Go RT-PCR Bead Protocol

Ready to go beads (RT-PCR) are premixed, predisposed reactions for performing RT-PCR. These beads are stable at room temperature and each bead contains all reagents except primer and template. This bead allows for a one-step RT-PCR reaction of 50μl in a single tube. Kit also contains control beads.

Materials:

Supplier is Pharmacia Biotech, catalog # 27-9267-01 (.2ml tubes/plate for 96 reactions). Storage: Room temperature. Wear gloves at all times while handling beads and always use sterile, filter pipette tips. RT-PCR bead .2ml tubes come in strips of 8 in a 96 well plate covered with a top paper layer over foil.

Control Mix: 5 control beads are included in the kit in red .5 ml microfuge tubes (1 ng of rabbit globulin mRNA and 8 pmol of two globulin specific PCR primers). These beads can be used to evaluate the performance of the RT-PCR Beads by adding the rehydrated control mix to a tube of RT-PCR Beads and performing RT-PCR along with samples. The product size is 550bp.

Procedure A: Handling of 0.2 ml Tubes/Plate

1. Remove plate from pouch, hold firmly and gently tap tray against the work bench to get the beads to the bottom of the tubes.
2. Slowly pull back the white paper (which is the top layer) from the corner to reveal the perforated foil layer at the first cut closest to the holes.
3. Hold down the edge of the bottom foil seal and carefully remove the top paper layer and discard. (If foil later is accidentally removed, reseal the unused tubes with the strip bubble caps that are provided.) Store unused reactions in the pouch with desiccant.
4. Decide the number of tubes needed and carefully cut through the paper and the plastic strip that connects the tubes. Slowly remove the outer perforated edge of all the tubes that will be used in the strip.
5. Gently push the needed tubes up from the bottom of the tray. Gently rock the strip of tubes until they come free from the plate. Be careful not to remove the foil seal from the tubes when pushing out the strip.
6. Place tubes in appropriate holder (snap apart one for the negative control and place them in the 9700 red rack and set the negative control tube on other side of tray).
7. Place the plate and other unused tubes into the resealable pouch with desiccant.

Procedure B: One-Step Protocol for RT-PCR

1. Check to make sure that all the beads are visible at the bottom of the tube. If necessary, tap the tubes against a hard surface to force each bead to the bottom

of the tube. Take out a strip of caps from the box and cut appropriate number to go with sample tubes.

2. Place the tubes on ice (cold block). Use one tube as a negative control.

3. Determine the total volume of RNA and primers that will be used in an RT-PCR reaction so that you can determine the volume of water in which to dissolve the bead. (For now the concentrations we use are as follows: template=11μl of RNA extract; 1 μl of 30 pmol RT primer; and .5 μl of 30 pmol PCR primers).

4. Remove foil and rehydrate the negative control bead with 48 μl of room temperature DEP-C water.

5. To the side of each sample tube add 37 μl of room temperature DEP-C water and let the beads dissolve for 5 minutes (This is to make sure that all the components are totally resuspended in solution). Flick gently with finger or pipet gently up and down and replace on ice. DO NOT vortex because it may cause foaming.

6. Add respective primers (1 μl of 30 pmol of RT primer and .5 μl of 30 pmol PCR primers) and transfer plate to the PCR room. Here you will add your respective templates (11 μl of RNA extract) to the proper sample tubes.

7. For the positive control reaction, add 50 μl of DEP-C treated water to the Control Mix Bead. Transfer the entire contents of the red tube to a tube containing an RT-PCR Bead. (*Note: we do NOT do this every time we PCR. This is done usually for the first reaction out of every new box of beads for QC).

8. Apply bubble caps to the tubes and make sure the fit is tight.

9. Transfer the reaction to a pre-warmed thermocycler where it will incubate at 42° for 30 minutes to complete the RT, heat to 95° for 5 minutes to inactivate the residual reverse transcriptase enzyme, and cycle 40 times to complete the PCR amplification.

Note: Step 9 is pre-programmed in the thermocycler as one program and set according to anneal temp variations. An example program looks like this: 42° X 30 mins (RT

step), 95° X 5 mins (Denature step), 94° X 30 sec. 57° X 30 sec. 72°X 1:15 sec (these three cycle 40 times), then 72° X 10 mins, and cool at 4°∞.

PCR Beads

(From Pharmacia Biotech; Cat # 27-9553-01; .2ml tubes/plate; 96 reactions) Storage: Room temperature. Wear gloves at all times while handling beads and always use sterile, filter pipette tips.

Procedure A: Handling of 0.2 ml PCR Tubes/Plate

These beads are the same as the RT-PCR beads except that they contain enough necessary reagents, except primers and template, to perform a 25μl PCR reaction instead of a 50 μl reaction like the RT-PCR bead. These beads are ideal for performing simple, room temperature PCR reactions without making master mixes and allows the use of beads for secondary PCR where RT reagents are not necessary.

1 Bead 25μl Reaction Method

1. The plates are packaged the same as the RT-PCR bead plates, so they are handled the same way (refer to Procedure A: Handling of 0.2 ml Tubes/Plate in RT-PCR instructions). In order to continue with a nested reaction using the PCR beads you must remember to adjust your primer and template volumes accordingly to fit a 25 μl reaction.
2. Make sure that the bead is at the bottom of the tube.
3. Add 21.5μl of room temperature distilled H2O to dissolve the bead. Then add .5 μl of each PCR primer. Transfer the reaction plate to the PCR room where you will then add 2.5 μl of your specific template to each sample tube. (*Note: for easier volume control, your PCR primers for this step are 15 pmol concentration).
4. Apply caps and make sure that they are tight.
5. Place rack on prewarmed, preprogrammed thermocycler and let amplify for 40 cycles.

NOTE: The following method is an alternative but is not what we currently use.

2 Bead 50 μl Reaction Method

1. The plates are packaged the same as the RT-PCR bead plates, so they are handled the same way (refer to Procedure A: Handling of 0.2ml Tubes/Plate in RT-PCR instructions) except that twice as many beads are going to be needed. In order to continue with a nested reaction using the PCR beads, two beads must be added to one single tube per reaction to equal 50 μl reaction volume.
2. After placing the appropriate number of tubes (including negative control) in a rack, take a second tube, turn it upside down on top of one of the designated tubes and lightly flick to dislodge bead so that it will drop into the bottom of the designated tube so that it now contains two. Do this for each tube set up for each reaction.
3. Make sure that both beads are at the bottom of the tube.
4. Add 44 μl of room temperature distilled H2O to dissolve the bead. Then add .5 μl of each PCR primer and 5 μl of your specific template to each tube.
5. Apply caps and make sure that they are tight.
6. Place rack on prewarmed, preprogrammed thermocycler and let amplify for 40 cycles.

Note: For our PCR reactions we amplify for 40 cycles. The parameters are the same except the anneal temp varies according to primers used. An example of a typical program goes like this: 94°X 5 minutes (denature), 94°X 30 sec, 57°X 30 sec, 72° X 1:15 sec, do this cycle X 40, then 72° X 10 mins, 4°X ∞.

S.O.P. (Quick Method cDNA/PCR for Genotyping) IV

RT-PCR READY TO GO BEAD PROTOCOL 1 step method

QUICK GLANCE REFERENCE SHEET

1. Remove pouch from box, wipe down and place in the hood.
2. Open pouch and carefully remove bead tray. Tap tray gently against the floor of the hood to make sure all beads are at the bottom of the tubes.

3. Slowly pull back the white paper (if still attached) to reveal the perforated foil layer.

4. Decide the number of tubes needed (add one for a negative control) and cut through paper and plastic strip that connects the tubes. Gently remove the tubes needed by rocking from the plate and place them on the 9700 red rack on the cold block.

5. Remove the foil and replace with caps. Make sure all beads are visible at the bottom of the tubes.

6. Add 37 μl of dep-C H2O to the side of each tube and let beads dissolve for 5 minutes. Flick gently or pipet up and down and replace on ice. Add 48 μl of H2O to the negative control tube.

7. Add respective primers (1μl of 30 pmol RT primer and .5 μl of 30 pmol PCR primers) and recap tubes. Move rack to the PCR/Thermocycler Room and add 11μl of the respective template to each tube.

8. Transfer reaction to pre-warmed thermocycler and incubate for the RT-PCR program. (These programs have the RT incubation settings and the PCR settings linked together for a continuous flow from cDNA to NlPCR.)

PCR STEP:

1. The steps are the same except that the volume for the PCR beads is 25 μl instead of 50 μl reactions.

2. After setting up your beads, add 21 μl of room temperature Dep-C H2O to dissolve the beads. Add 21.5 μl of Dep-C H2O to the negative control tube. Next, add 1 μl each of 15 pmol primer stocks (this is so that you can add .5 μl if 15 pmol instead of .25 μl of 30 pmol.)

3. Transfer reaction tubes to RT/PCR room and add .5μl of specific template to each tube. Reapply tops and place rack on prewarmed, preprogrammed thermocycler and let amplify for 40 cycles at the appropriate anneal temperature.

Cycle Sequencing for MicroGeneBlaster OpenGene System (Visible Genetics, Inc.) Using Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham LIFE SCIENCE cat #: RPN 2538)

1. Add 2 μl of ddNTP/Enz/Buffer reaction mix to 0.2 ml strip tube, on cold block, in the order of A C G T.
2. To a 0.5 ml tube add the following reagents and mix well by vortex:

   PCR DNA template (>200 ng)

   Cy 5.5 labeled primer (5 pmol) 1.5μl

   D-H2O

   Total Volume: 26.5μl
3. Aliquot 6.0μl of template/primer mix to the tube wall of each A C G T 0.2 ml tubes in step one.
4. Start the thermocycler program and pause program at 94°C.
5. Mix sequencing reaction by vortex and swinging the plate downward, then place onto the thermocycler and resume program.

   Parameters for Cycle Sequencing using Perkin-Elmer 9700 Thermocycler and Max-mode software:

   Initial denature:

   94 °C 2 minutes

   40 cycle:

   94 °C 20 seconds

   58 °C 20 seconds

   70 °C 30 seconds

   Extension:

   70 °C 5 minutes

   Hold:

   4 °C
6. When cycling is completed, add 6 μl of the red loading buffer to each 0.2 ml tubes after program reaches 4 °C.

7. Heat samples to 80 °C for 3 minutes, snap-chill by placing sample on cold-block in -20°C for 2 minutes.

8. Load 2 μl of each sample to each corresponding lane on MicroGeneBlaster automated sequencer using GeneObjects v3.0 (Visible Genetics, Inc.)

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CLAIMS

1. A method for detecting a polymorphic mutation effective to act as a predictor of subsequent induction of a drug-resistance mutation at a defined site in a genetic sequence of interest obtained from a source of sample, comprising the steps of:

(a) obtaining a test sample containing the genetic sequence of interest from the source, said genetic sequence comprising a plurality of nucleotide bases; and

(b) comparing the bases in a region of the genetic sequence of interest in the test sample located on at least one side of and near the defined site with the same bases in a standard sequence or a sequence determined from a prior sample obtained from the same source, wherein a difference in the bases between the test sample and the standard sequence or prior sample from the same source is indicative of the presence of polymorphic mutation effective to act as a predictor of subsequent induction of a drug-resistance mutation and thus of an increased risk of subsequent induction of a drug-resistance mutation at the defined site.

2. The method of claim 1, wherein the bases are compared by sequencing of the bases in a region of the genetic sequence of interest in the test sample located on at least one side of and extending for 3 to 138 bases from the defined site and comparing the result with a standard sequence.

3. The method of claim 1, wherein the bases are compared based on the ability to bind a hybridization probe.

4. The method of any of claims 1 to 3, wherein the genetic sequence of interest is derived from the genome of human immunodeficiency virus.

5. The method of claim 4, wherein the drug resistance mutation is a mutation at codon 214 of the HIV reverse transcriptase gene.

6. The method of claim 5, wherein the bases of codon 214 are compared with a standard sequence CTT.

7. The method of claim 4, wherein the drug resistance mutation is a mutation at codon 36 of the HIV protease gene.

8. The method of claim 7, wherein the bases of codon 37 are compared with a standard sequence AAT.

9. The method of claim 7, wherein the bases of codon 35 are compared with a standard sequence GAA.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/11959

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/02, 21/04
US CL :435/6; 536/23.1, 23.72, 24.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 23.72, 24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, BIOSIS, EMBASE, MEDLINE, AIDSLINE, polymorphism, drug resistance, muttion, HIV, virus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRIGAN ET AL. Significance of Amino Acid Variation at Human Immunodeficiency Virus Type 1 Reverse Transcriptase Residue 210 for Zidovudine Susceptibility. J. Virology. September 1996, Vol. 70, No. 9, pages 5930-5934, especially Abstract and Table 1.	1-6
Y	SHIOTA ET AL. Mutation of the thymidine kinase gene in a ganciclovir-resistant strain of herpes simplex virus type 1 (HSV-1) detected by pcr-sscp and pcr-direct sequencing. Biomedical Research. 1996, Vol. 17, No. 1, pages 9-14, especially Abstract and Tables 1 and 2.	1-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 16 AUGUST 1999	Date of mailing of the international search report 13 SEP 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HANKYEL T. PARK, PH.D. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/11959

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCARPELLINI ET AL. Detection of Rifampin Resistance by Single-Strand Conformation Polymorphism Analysis of Cerebrospinal Fluid of Patients with Tuberculosis of the Central Nervous System. J Clinical Microbiology. November 1997, Vol. 35, No. 11, pages 2802-2806, especially Abstract and Table 1.	1-9